



**UNIVERSITI PUTRA MALAYSIA**

**IN VITRO SELECTION IN WHEAT TO RECOVER  
MUTANTS WHICH OVERPRODUCE  
LYSINE AND THREONINE**

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**FPV 1991 6**

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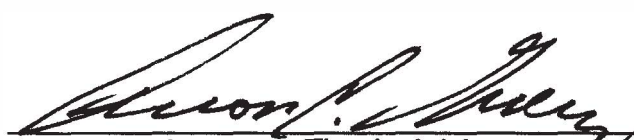
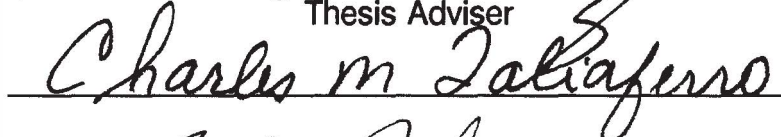
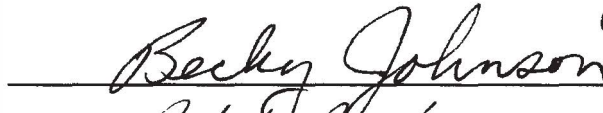
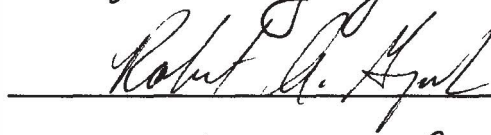

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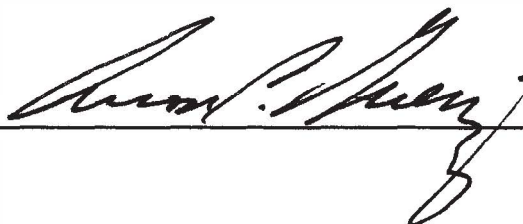
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Scope of Study: Lysine and threonine are the first and second limiting amino acids in wheat for proper human and non-ruminant animal nutrition. The objective of this study was to utilize in vitro selection to recover mutations for enzymes regulating lysine and threonine biosynthesis in wheat. High concentrations of lysine and threonine cause feedback inhibition to regulatory enzymes in the aspartate family of amino acids which lysine and threonine are members. This results in growth inhibition due to methionine starvation. Specific objectives were: 1) study the effect of lysine, threonine or methionine on seed development with wheat spike culture, 2) study the effect of lysine and threonine (LT) on wheat callus growth, and 3) recover mutants which overproduce the essential amino acids lysine and threonine through direct and stepwise in vitro selection strategies.

Findings and conclusions: Results from wheat spike culture experiment indicated that spikes cultured in medium containing 1 mM lysine and threonine had 13% reduction in protein percentage. Inclusion of methionine with lysine and threonine enhanced grain development by increasing seed number per spike. Growth of wheat callus on media containing 1, 2, 4 and 8 mM LT was significantly inhibited. Plants were regenerated from callus growing on 1, 2, and 3 mM LT media. Seven R<sub>1</sub> plants contained significantly higher concentrations of total lysine or threonine in the seed. The average increase for total lysine and threonine was 43% and 63%, respectively. Two R<sub>2</sub> plants had an average increase of 150% in total lysine and another two plants had an average increase of 60% in total threonine. These genetic stocks should be useful in developing wheat cultivars with improved nutritional quality.

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## INTRODUCTION

Each chapter in this thesis is a manuscript to be submitted for publication in Crop Science, a Crop Science Society of America publication.

CHAPTER I

THE EFFECT OF LYSINE, THREONINE OR METHIONINE

ON SEED DEVELOPMENT IN WHEAT

SPIKE CULTURE

**The Effect of Lysine, Threonine or Methionine on Seed  
Development in Wheat Spike Culture**

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## ABSTRACT

Lysine and threonine cause feedback inhibition to aspartokinase, dihydropicolinate synthase, and homoserine dehydrogenase which result in growth inhibition due to lack of methionine. This research was conducted to study the effect of lysine, threonine and methionine on wheat seed development and amino acid content. Wheat spikes were grown in liquid media with three different concentrations of lysine, threonine or methionine. Concentrations used were 1 mM lysine and threonine (LT), 1 mM lysine, threonine and methionine (LTM) and without lysine, threonine or methionine (0 LTM). Spikes were cultured from anthesis for a period of 6 wk. Seed number per spike, seed weight and amino acid composition were analyzed. No significant effects on seed number per spike and seed weight were observed for spikes cultured in 1 mM LT medium. Spikes cultured in 1 mM LT medium had a significant reduction in total concentration of ten amino acids. The reductions in the 10 amino acids lead to a significant 13% reduction in protein percentage. Inclusion of methionine with lysine and threonine overcame the inhibitory effect of lysine and threonine on accumulation of 7 amino acids. Addition of lysine, threonine and methionine also enhanced grain development by increasing seed number per spike. Inclusion of methionine does not overcome the significant reduction in total alanine, glutamate and glycine. It did improve the concentration of cysteine, isoleucine and valine when compared to the control.



## INTRODUCTION

Wheat spike culture is a useful tool in studying grain development.

Spike are easily cultured in liquid medium consisting of basic nutrients and other elements of interest. Spike culture has been used to study the effects of various treatments on grain development. Wheat spike culture medium containing sucrose, amino acids, salts and minor organic elements was first developed by Donovan and Lee (1977). Their study indicated that media deficient in sucrose or amino acids lead to slow accumulation of dry weight. They also observed that radioactively labelled amino acids supplemented in the liquid media are rapidly incorporated into protein. The use of spike culture for studying grain development is very promising. Singh and Jenner (1983) found that wheat spikes can be cultured in liquid media from anthesis until grain maturity.

Amstrong et al. (1987) performed wheat spike culture in Murashige and Skoog media with 3% sucrose from anthesis for a period of 10 d. They reported a 3 fold increase in distal floret seed number of cultured spikes as compared to the intact control. Spike culture has also been used in barley to study protein and carbohydrate accumulation in normal and high-lysine barley (Manther and Giese, 1984). Corke and Atsmon (1988) used spike culture to study the effect of nitrogen nutrition on endosperm protein synthesis in wild and cultivated barley.

The inhibitory effect of lysine and threonine (LT) on the growth of callus

tissue and regulatory enzymes for the aspartate family of amino acids has been well documented (Furuhashi and Yatazawa, 1970; Wong and Dennis, 1973; Green and Phillips, 1974; Sakano and Komamine, 1978; Bright et al., 1978). However, to date no work has been conducted to study the effect of lysine and threonine on seed development. Therefore, this research was conducted to study the effect of lysine, threonine and methionine on seed development and their effect on the amino acid composition of the seed by utilization of spike culture.

## MATERIALS AND METHODS

### *Spike Culture*

'Bobwhite' wheat was used for this study. Plants were grown in the greenhouse at 20°C with a 14 h photoperiod. The procedure employed was similar to the one described by Armstrong et al. (1987). After anthesis, spikes were removed from the plant by cutting the stem underwater just above the flag leaf. The peduncle portion of the spike was surface sterilized in 30% (v/v) clorox with 60  $\mu\text{L L}^{-1}$  Triton-X. One cm from the base of the peduncle was cut-off under sterilized water after sterilization. The basic medium consisted of Murashige and Skoog (MS) inorganic salts (Murashige and Skoog, 1962), Gamborg B5 vitamins (Gamborg and Eveleigh, 1968), 3% sucrose, 0.15  $\text{g L}^{-1}$  L-asparagine, and 0.25  $\text{g L}^{-1}$  each of benlate, ampicillin and streptomycin. Three treatments of combinations of lysine, threonine or methionine were used for this study. The treatments were: 1) control without lysine, threonine or methionine (OLT), 2) 1 mM lysine plus threonine (LT), and 3) 1 mM lysine, threonine and methionine (LTM). Each spike was cultured in 100 ml of medium in a 125 ml Erlenmeyer flask. It was held in place by a sterilized split foam plug. Each flask was considered a replication. Two experiments were conducted. In the first experiment each treatment was replicated 6 times while in the second experiment each treatment was replicated 5 times. Every 2 wk the spike was transferred into similar fresh medium. Spikes were cultured for a period of 6 wk. After 6 wk spikes were harvested and seed yield, seed spike<sup>-1</sup>

and average seed weight were determined. Data were analyzed by analysis of variance.

#### ***Analysis of total amino acids***

One-tenth gm wheat meal from each seed sample was placed in a 6 x 50 mm pyrex disposable culture tube. One thousand nmol norleucine was added as an internal standard to each sample. Samples were hydrolysed with 6 N HCl for 24 h at 100°C under nitrogen. After hydrolysis, samples were dried by vacuum centrifugation. Samples were redissolved with 3 washes of 500 ul of deionized water and filtered with a 0.45 um nylon filter. Further preparation and derivatization steps were adapted from the Pico-Tag™ procedures (Cohen et al., 1984). Amino acid derivatives were separated and quantified by reverse-phase high performance liquid chromatography (Heinrikson and Meredith, 1984). Two determinations were performed for each sample.

#### ***Analysis of free amino acids***

Twenty seeds from each spike of the same treatment were pooled and ground by mortar and pestle. Experiments 1 and 2 were analyzed separately. Approximately 0.1 gram meal from each seed sample was place in a 5 ml tube. An internal standard of 50 nmol norleucine was added into each tube. Amino acids were extracted with 12:15:3 (v/v/v) methanol, chloroform and water (Bieleski and Turner, 1966). From each sample, 2.5 ml of methanol-water phase (top layer) was removed and transferred to a 5 ml cryotube. The

samples were then dried by vacuum centrifugation (Speed-Vac, Savant Instruments). Samples were then subjected to mild acid hydrolysis with 2 N HCl at 100°C for 2 h to convert asparagine and glutamine to aspartic and glutamic acid, respectively. After acid hydrolysis samples were dried by vacuum centrifugation. The sample was then resuspend in 400 ul of 2:1 water:methanol (v/v) and then transferred to a 10,000 m.w. ultrafiltration device (Millipore Inc.). One hundred ul of the filtrate was sampled and dried. Other preparation and derivatization procedures were similar to those described above for total amino acids analysis. Two determinations were conducted for each sample.

## RESULTS

### *Spike culture*

Spikes cultured in media containing 1 mM LTM showed significant increases in seed number spike<sup>-1</sup> when compared to the control (Table 1). However there was no significant difference between the control and 1 mM LT for seed spike<sup>-1</sup>. For average seed weight, no significant differences were observed between treatments and the control.

### *Total amino acid concentration of seed*

An analysis of variance on combined data from experiment 1 and 2 showed significant treatment by experiment interactions for total amino acids (Table 2) and mole percentage (mol%) (Table 3), therefore each experiment was analyzed separately.

Experiment 1. Total amino acid concentration of seed (Table 4) from spikes cultured on 1 mM LT had a significant reduction in aspartate, cysteine, glutamate, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, tyrosine, valine and total amino acid concentration when compared to the control. For mol%, significant reductions in cysteine and lysine were noted, while significant increases in mol% for alanine, leucine, phenylalanine, proline and threonine were observed.

For spikes cultured on 1 mM lysine, threonine and methionine (LTM) significant increases in alanine, aspartate, isoleucine, leucine, lysine,

**Table 1.** Combined data for seeds per spike, seed weight and protein percentage for spikes cultured in media supplemented with lysine, threonine or methionine.

Treatment	Seed spike <sup>-1</sup>	Seed wt.	Protein	Total AA
		-gm-	-%-	-nmol mg <sup>-1</sup> -
Control	30.0	0.0178	21.5	1705.1
1 mM LT	33.0	0.0174	18.6*	1477.3*
1 mM LTM	41.0*	0.0180	21.6	1720.6
LSD (0.05)	6.9	NS	1.2	90.0

\* Significantly higher or lower than control at  $P \leq 0.05$

**Table 2.** Combined analysis of variance for total amino acids.

Source	d.f.	Amino Acid								
		Ala	Arg	Asx	Cys	Glx	Gly	His	Ile	Leu
Treatment (Trt)	2	**	NS	*	*	*	**	NS	**	**
Experiment (Exp)	1	NS	NS	NS	NS	NS	*	NS	*	NS
Determination (Exp)	2	NS	NS	NS	NS	NS	NS	NS	NS	NS
Trt *Exp	2	**	NS	NS	**	**	**	NS	**	**
Error	4	-	-	-	-	-	-	-	-	-

\*, \*\* Significant at  $P \leq 0.05$  and  $P \leq 0.01$ , respectively

NS  $P > 0.05$



**Table 2.** (Continued)

Source	d.f.	Amino Acid								Total
		Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val	
Treatment (Trt)	2	NS	*	NS	*	NS	*	NS	**	**
Experiment (Exp)	1	**	NS	NS	**	NS	NS	NS	**	**
Determination (Exp)	2	NS	NS	NS	NS	NS	NS	NS	NS	NS
Trt *Exp	2	**	*	**	**	NS	**	*	**	**
Error	4	-	-	-	-	-	-	-	-	-

\*, \*\* Significant at  $P \leq 0.05$  and  $P \leq 0.01$ , respectively  
 NS  $P > 0.05$